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The reaction of nitrite with the haemocyanin of *Astacus leptodactylus*

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The reaction of nitrite at pH 5.7 with deoxyhaemocyanin of *Astacus leptodactylus* yielded methaemocyanin in two one-electron steps, as nitrite was reduced to NO. This methaemocyanin could be almost fully regenerated by an anaerobic treatment with $HONH_2$, in contrast with the methaemocyanin prepared with H_2O_2. A destruction of active sites on treating oxyhaemocyanin with $HONH_2$ explains the partial regeneration of methaemocyanin under air, as traces of H_2O_2 are formed in the autoxidation of $HONH_2$. The reaction rate of nitrite with deoxyhaemocyanin is almost 15 times that with oxyhaemocyanin. The slope of -1.0 for the logarithm of the pseudo-first-order rate constants plotted against pH indicates that HNO_2 is the reacting species. Methaemocyanin was e.p.r.-undetectable, but a binuclear signal was observed at $g = 2$ on binding nitrite to methaemocyanin. This signal disappeared with a pK_a of 6.50, suggesting that a μ-aquo bridging ligand, which can be replaced by nitrite, is deprotonated to a μ-hydroxo bridging ligand, which resists substitution by nitrite. The intensity of this triplet e.p.r. signal allowed the determination of the association constant of nitrite to the active site of *Astacus* methaemocyanin and yielded a value of 237 M^{-1} at pH 5.7. The interpretation by some authors of nitrosylhaemocyanin as a nitrite derivative of semi-methaemocyanin is contradicted by this rapid reaction of nitrite with copper(I) in deoxyhaemocyanin and in semi-methaemocyanin and by the low binding constant of nitrite to the active site of methaemocyanin.

INTRODUCTION

Haemocyanins (Hcs), the copper-containing respiratory proteins of arthropods and molluscs (Préaux & Gielens, 1984), bind one O_2 molecule per active site (Cu^ICu^I) as peroxide to $Cu^{II}Cu^{II}$ (Freedman *et al.*, 1976). Arthropodan Hcs contain one active site per 75000 M_r. For the Hc of the spiny lobster *Panulirus interruptus* (presumably the deoxy derivative, as the crystals had been obtained at pH 4.5, where the p_{50} is high) six histidine residues were identified as the metal ligands by X-ray diffraction at a resolution of 0.32 nm (Volbeda & Hol, 1986).

A partial amino acid sequence has been reported for subunit *b* of the Hc of the river crayfish *Astacus leptodactylus* (Schneider *et al.*, 1986). A comparison with the sequences of subunits *a–c* of *Panulirus* Hc indicated conserved copper ligands (in the numbering for subunit *a* of *Panulirus* Hc): His-194 and His-198 on α-helix 2.1, His-224 on α-helix 2.2 for Cu_A, and His-344 and His-348 on α-helix 2.5, His-384 on α-helix 2.6 for Cu_B in the centre of the second of the three protein domains (Soeter *et al.*, 1986).

Astacus methaemocyanin (MetHc), prepared with NO (101 kPa) or with nitrite, could be regenerated to the extent of about 70% with $HONH_2$ under air (Witters *et al.*, 1986*a*), in contrast with MetHc obtained by the classical treatment of DeoxyHc with H_2O_2 (Felsenfeld & Printz, 1959), which could not be regenerated under these conditions.

The preparation by the action of nitrite of a MetHc of *Astacus* that could almost fully be regenerated by an anaerobic treatment with $HONH_2$ is reported in the present paper, together with a quantitative analysis of the binding of nitrite to its active site and an investigation of the reaction of nitrite with DeoxyHc and with OxyHc.

MATERIALS AND METHODS

The haemolymph of *Astacus*, collected by inserting an injection needle between abdomen and carapace, was treated with an equal volume of 0.1 M-sodium acetate buffer, pH 5.7, containing 0.13 M-EDTA in order to prevent coagulation. The solution was dialysed against distilled water to precipitate the lipoproteins and glycoproteins. After centrifugation at 3000 *g* ($r_{av.}$ 10 cm) for 1 h, the supernatant was dialysed against 0.1 M-sodium acetate buffer, pH 5.7, and stored at 4 °C.

Protein concentrations were determined in a Perkin–Elmer 554 spectrophotometer by using $A^{1\%}_{1\,cm,278}$ 12.36 in 50 mM-sodium tetraborate buffer, pH 9.2 (Pilz *et al.*, 1980). The Cu–O_2 absorption band was used to determine the amount of OxyHc ($A^{1\%}_{1\,cm,338}$ 2.16 in 0.1 M-sodium acetate buffer, pH 5.7). These measurements were always carried out after re-equilibration at the air.

The reaction of nitrite with OxyHc under 600 kPa O_2 in a stainless-steel bomb was monitored in a Cary 16 spectrophotometer (Varian, Monrovia, CA, U.S.A.). Spectrophotometric experiments in the presence of

Abbreviation used: Hc, haemocyanin.

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nitrite were corrected for the absorbance of nitrite (ϵ_{338} 21.8 $M^{-1} \cdot cm^{-1}$ in 0.1 M-sodium acetate buffer, pH 5.7).

For the study of the influence of pH on the formation of MetHc and on the binding of nitrite to MetHc, the Hc and the MetHc of *Astacus* were dialysed against the appropriate buffer solutions (I 0.1): sodium acetate/acetic acid, pH 5.0–5.7; Na_2HPO_4/NaH_2PO_4, pH 6.0–7.0; Tris/$HClO_4$, pH 7.0–8.0; $Na_2B_4O_7/HClO_4$, pH 8.0–9.2. Cl^- ions were avoided as they had been shown to enhance the binuclear e.p.r. signal at $g = 4$ of *Helix pomatia* MetHc (Witters *et al.*, 1981).

The chemicals were of analytical grade: $CuSO_4,5H_2O$ and $(HONH_3)_2SO_4$ from Merck (Darmstadt, Germany) and $NaNO_2$ from UCB (Brussels, Belgium).

Manometric measurements were carried out in a Warburg apparatus (Braun, Melsungen, Germany); for experiments with DeoxyHc the reaction vessel was flushed with N_2 (A28; L'Air Liquide Belge, Liège, Belgium).

The gaseous products of the reaction of nitrite with DeoxyHc were analysed by means of a modified Micromass type 8-80 mass spectrometer (Vacuum Generators, Hastings, Sussex, U.K.), equipped with an 8 cm 80° magnetic analyser and an enclosed ionization source. A vacuum stage was connected to the latter, consisting of a CCT 100 liquid-N_2 trap (Vacuum Generators) and a combination of an EO_4 oil diffusion pump with an E2M8 rotary pump (Edwards, Newcastle, U.K.). With a pressure in the analyser of 1.33 mPa, gaseous samples were taken from the reaction vessel (volume 142 ml) and introduced directly into the ionization source by means of an ELF precision needle valve (Brooks, Hatfield, PA, U.S.A.), yielding a sampling flow of about 1 ml/min under s.t.p. conditions. He (N56; L'Air Liquide Belge) was used as a carrier gas. Absolute sensitivities for trace compounds such as NO were derived from a calibration of the instrument with a certified gas standard (1.03 ± 0.02% NO in He; L'Air Liquide Belge). The measurements were carried out at the following instrument settings: an electron energy of 40 eV, a filament emission current of 40 μA, and an acceleration and ion-multiplier voltage of 3 kV each. The accuracy of the measurements was about ±2%.

The e.p.r. spectra were recorded on a Varian E-109 spectrometer (Palo Alto, CA, U.S.A.) at 133 K at a microwave frequency of 9.107 GHz with 100 kHz field modulation at a modulation amplitude of 1 mT and a microwave power of 30 mW. The signal intensity was determined by double integration with a Hewlett–Packard 9825A calculator (Loveland, CO, U.S.A.). A solution of 1 mM-$CuSO_4$/10 mM-$HClO_4$/2 M-$NaClO_4$ was used as a standard for integrating the mononuclear and binuclear signals at $g = 2$.

RESULTS AND DISCUSSION

pH-dependence of the reaction

As an indication for the formation of MetHc the decrease of the absorbance at 338 nm of the Cu–O_2 band was measured as a function of time after the addition to DeoxyHc and to OxyHc of $NaNO_2$ in a molar ratio to copper, R, of 110. The reaction was pseudo-first-order with respect to Hc. The logarithm of the pseudo-first-order rate constant decreased linearly with pH with a slope of −1.08 for DeoxyHc, −1.04 for Hc under air (7.7% DeoxyHc, 92.3% OxyHc at pH 5.7, based on a p_{50} value of 13.3 mmHg), and −0.91 for OxyHc (99.7% at pH 5.7, similarly calculated) under 600 kPa O_2 (Fig. 1). No reaction was observed at pH 8.2.

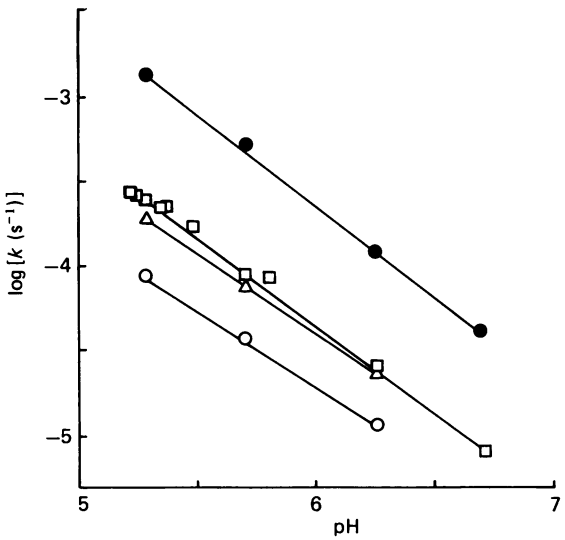

Fig. 1. Plots of the logarithm of the pseudo-first-order rate constants k as a function of pH for the reaction at room temperature of 2.6 mM-$NaNO_2$ with *Astacus* Hc (about 1.0 mg/ml, about 24 μM-Cu)

●, DeoxyHc under 101 kPa N_2; ○, OxyHc (99.7% at pH 5.7) under 600 kPa O_2; □, Hc under air (7.7% DeoxyHc, 92.3% OxyHc at pH 5.7); △, values for Hc under air calculated from those obtained for DeoxyHc and OxyHc.

The faster reaction rate of DeoxyHc with nitrite, about 15 times that of OxyHc under 600 kPa O_2, could at least partially be linked to the co-ordination number of copper, which is lower for Cu(I) in DeoxyHc than for Cu(II) in OxyHc, as determined by X-ray absorption spectrometry (Co & Hodgson, 1984). The rate constants for the reaction under air, calculated from those for DeoxyHc and OxyHc and from their concentrations obtained from the p_{50} values, yielded a slope of −0.92 (Fig. 1).

Slopes of about −1.0 in the plot of $\log k$ against pH (Fig. 1) suggest that HNO_2 (pK_a 3.15 at 25 °C) is the reacting species. The diffusion of a small and neutral molecule to the active site could be the rate-limiting step. The formation of MetHc in the reaction of the molluscan OxyHc with fluoride and azide, which correspond to weak acids with pK_a respectively 3.20 and 4.65 at 25 °C, was also enhanced by lowering the pH (Witters & Lontie, 1975). A similar observation with a slope of −0.88 has been made for the reaction of nitrite with human deoxyhaemoglobin (Doyle *et al.*, 1981).

After removal of nitrite by dialysis against an appropriate buffer, the e.p.r. measurements showed only a small mononuclear signal at $g = 2$ (from 5–10% of the copper), probably due to broken copper pairs (Fig. 2, trace A).

Formation of NO

The reaction of nitrite with DeoxyHc, carried out in a Warburg apparatus, revealed the liberation of gaseous products. Their nature was investigated by mass spectro-

Table 1. Signal intensities and product concentrations, determined by m.s. after 2 h reaction of 59.5 mM-nitrite in 0.1 M-sodium acetate buffer, pH 5.7, with 12 ml of DeoxyHc and of MetHc (26 mg/ml, 0.61 mM-Cu) under 101 kPa He at room temperature

m/z	Molecular ion	Signal intensity (nA) DeoxyHc	MetHc	Difference	Concentration (p.p.m.)
28	N_2^+	31.7	27.6	4.1	40
30	NO^+	226.5	43.0	183.5	1300
32	O_2^+	1.9	2.7	−0.8	−5
44	N_2O^+ or CO_2^+	9.3	6.9	2.4	24
46	NO_2^+	0.1	$\ll 0.1$	0.1	1

metry (Table 1). Possible contributions to the observed mass-peak intensities from background sources could be accounted for by running a blank with MetHc, prepared in accordance with Felsenfeld & Printz (1959) by treatment of DeoxyHc (24.8 mg/ml, 0.587 mM-Cu) with H_2O_2 at $R = 10$. After 24 h reaction in 0.1 M-sodium acetate buffer, pH 5.7, the excess H_2O_2 was removed by dialysis against the same buffer. NO was the major product (> 95%); N_2, N_2O (or CO_2) and NO_2 were only minor components. The detected value of 1300 p.p.m. of NO corresponded to 6.99 μmol, which correlated favourably with 7.32 μmol of Cu in the DeoxyHc solution. According to Henry's Law less than 0.5% NO remained in solution under the experimental conditions.

In its reaction with DeoxyHc nitrite is thus reduced in a one-electron step to NO. As no Semi-MetHc, $Cu^I_ACu^{II}_B$ or $Cu^{II}_ACu^I_B$, was detected by e.p.r. at $g = 2$, the oxidation of the second Cu^I in the active site must have been much faster than that of the first.

The description of NitrosylHc as a nitrite derivative of Semi-MetHc (Hwang & Solomon, 1982) is already invalidated by this rapid reaction of nitrite with Cu^I in DeoxyHc and in Semi-MetHc.

No further reaction of *Astacus* MetHc with the liberated NO was observed. Moreover, no decrease of the O_2-binding capacity of Hc was noted after 24 h reaction of NO (1.04 kPa) with DeoxyHc at room temperature in 0.1 M-sodium acetate buffer, pH 5.7.

The oxidation–reduction potential of the system $NO_2^-/NO^\cdot$ ($E'_0 = 0.52$ V at pH 5.7 and 0.36 V at pH 7.0) sets an upper limit to the oxidation–reduction potential of the system MetHc/DeoxyHc.

The reaction of nitrite with OxyHc could produce either nitrate or NO:

$$Cu^{II}Cu^{II}O_2^{2-} + HNO_2 + H^+ \rightarrow Cu^{II}Cu^{II} + NO_3^- + H_2O \quad (1)$$

$$Cu^{II}Cu^{II}O_2^{2-} + 2HNO_2 + 2H^+ \rightarrow Cu^{II}Cu^{II} + O_2 + 2NO^\cdot + 2H_2O \quad (2)$$

The ready reaction of NO with O_2 unfortunately also leads to the overall stoicheiometry of eqn. (1):

$$2NO^\cdot + O_2 \rightarrow 2NO_2^\cdot \quad (3)$$

$$2NO_2^\cdot + 3H_2O \rightarrow NO_2^- + NO_3^- + 2H_3O^+ \quad (4)$$

Binuclear e.p.r. signal of the nitrite derivative of MetHc

Like OxyHc, the MetHc of *Astacus* is e.p.r. non-detectable, which can be ascribed to a reasonably strong super-exchange coupling of both copper ions in the active site by a bridging ligand. An endogenous protein ligand like tyrosine (Wilcox *et al.*, 1984) or an exchangeable exogenous ligand like the OH^- ion (Coughlin & Lippard, 1981) have been proposed. A phenolate bridging ligand was excluded for crustacean Hc by the X-ray-diffraction studies on *Panulirus* Hc (Volbeda & Hol, 1986) and by comparing model complexes with Hcs by resonance Raman spectroscopy (Lorösch *et al.*, 1986).

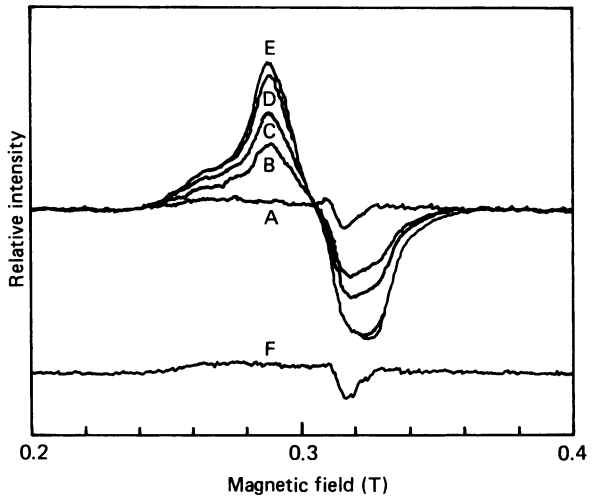

Fig. 2. E.p.r. signal at $g = 2$, measured at 133 K, as a function of time in the reaction at room temperature of 10.9 mM-$NaNO_2$ with *Astacus* MetHc (20.3 mg/ml, 0.48 mM-Cu), prepared with nitrite, in 0.1 M-sodium acetate buffer, pk 79 H 5.7

Trace A, blank; trace B, after 5 min reaction; trace C, after 15 min reaction; trace D, after 2 h reaction; trace E, after 18 h reaction; trace F, after removal of nitrite by dialysis. Receiver gain was 10000.

A broad binuclear e.p.r. signal at $g = 2$ has been observed with crustacean MetHc in the presence of nitrite at pH 5.7–5.9 (Gondko *et al.*, 1985; Witters *et al.*, 1986*b*). On adding nitrite ($R = 22.3$) at room temperature to *Astacus* MetHc prepared by the action of nitrite, a triplet signal at $g = 2$ was slowly formed, which reached a constant value after 18 h (Fig. 2, traces B–E). No half-field signal at $g = 4$ was observed. The binding of nitrite was reversible, as the broad triplet signal disappeared on dialysis against 0.1 M-sodium acetate buffer, pH 5.7 (Fig. 2, trace F).

The broad binuclear e.p.r. signal at $g = 2$ of the nitrite derivative of *Astacus* MetHc excludes a strong super-exchange coupling. This single isotropic signal, due to dipole–dipole interaction, and the absence of a half-field

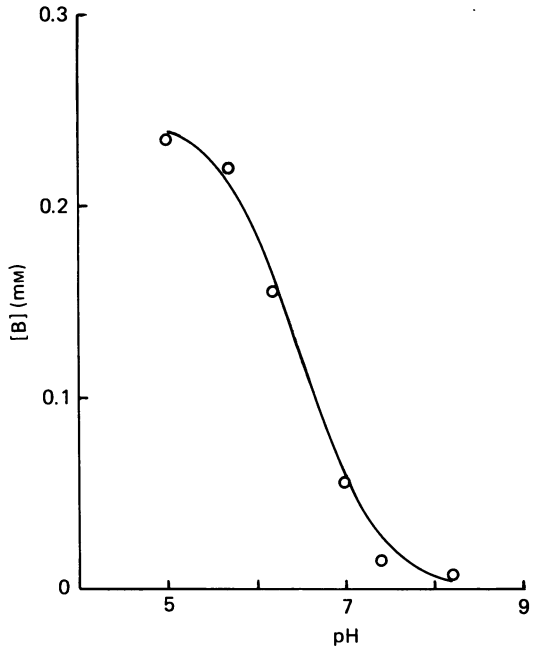

Fig. 3. Concentration of bound nitrite [B], deduced from e.p.r. signal intensities, to *Astacus* MetHc (25 mg/ml, 0.59 mM-Cu), prepared with nitrite, at room temperature as a function of pH at a nitrite concentration of 15.8 mM

The curve was calculated by the Henderson–Hasselbalch equation with a pK_a value of 6.50.

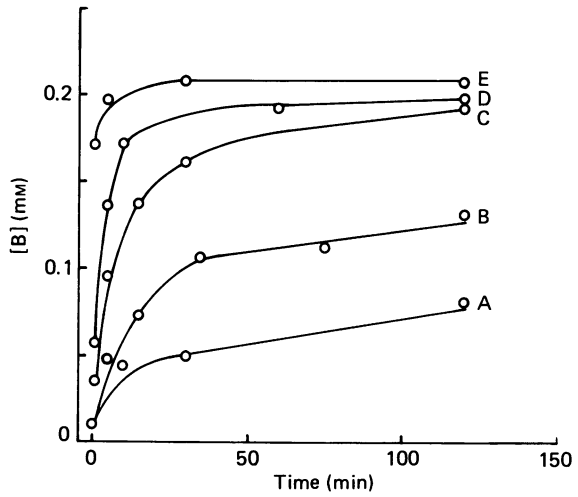

Fig. 4. Concentration of bound nitrite [B], deduced from e.p.r. signal intensities, to *Astacus* MetHc (20.3 mg/ml, 0.48 mM-Cu), prepared with nitrite, as a function of time at room temperature in 0.1 M-sodium acetate buffer, pH 5.7

Nitrite concentrations: trace A, 2.7 mM; trace B, 5.2 mM; trace C, 10.9 mM; trace D, 27.2 mM; trace E, 54.4 mM.

signal at $g = 4$ indicate a very weak zero-field splitting (Boas, 1984).

The pH-dependence of the binding at room temperature of nitrite to MetHc was measured by e.p.r. (Fig. 3). The intensity of the signal after 18 h, plotted against pH, followed the Henderson–Hasselbalch equation and yielded a pK_a value of 6.50, which could apply to a μ-aquo bridging ligand. By substitution with nitrite the super-exchange coupling is replaced by dipole–dipole interaction. The deprotonation of the bridging ligand leads to a μ-hydroxo structure with strong super-exchange, which cannot be substituted by nitrite. A similar decrease of the pK_a to about 7.4 of a water molecule bound to type-2 Cu(II) in *Rhus* laccase has been described (Andréasson & Reinhammar, 1979).

The integration of the signal at $g = 2$ yielded a value of 0.40 mM-Cu(II), corresponding to 0.20 mM detectable active sites (about 80%). At pH 5.7, however, according to the pK_a of 6.50 6% of the binuclear sites yield already no signal.

Binding of nitrite to MetHc at pH 5.7

The binding at room temperature of nitrite at increasing concentrations to MetHc in 0.1 M-sodium acetate buffer, pH 5.7, was monitored by e.p.r. as a function of time (Fig. 4). The final signal intensity increased with the nitrite concentration from $R = 1$ to 50. For $R = 100$ and 200 almost the same final signal was obtained, indicating a saturation of the active sites with nitrite. In contrast with molluscan MetHc (Verplaetse *et al.*, 1979), no NitrosylHc was formed.

In binding experiments the Scatchard and the Woolf graphs are used to determine the binding constant $K_{ass.}$ and the number of binding sites n. The concentration of bound nitrite [B] was derived from the intensity of the e.p.r. signal and based on the plausible assumption of the binding of one nitrite ion per active site. The concentration of free nitrite [F] was calculated from the initial nitrite concentration minus [B] (a rather unimportant correction with a low binding constant). The concentration of free active sites was given by the total concentration of active sites [P] (0.24 mM) minus [B].

$$\frac{[B]}{[F]([P]-[B])} = K_{ass.} \quad (5)$$

The degree of binding ν is given by:

$$\nu = \frac{[B]}{[P]} = \frac{n \cdot K_{ass.}[F]}{1 + K_{ass.}[F]} = n \cdot K_{ass.}[F] - \nu \cdot K_{ass.}[F] \quad (6)$$

Multiplying by [P] yields the Scatchard (1949) equation:

$$\frac{[B]}{[F]} = n \cdot K_{ass.}[P] - K_{ass.}[B] \quad (7)$$

According to a linearization procedure, proposed by Woolf in enzyme kinetics and also called the Hanes plot (Keightley *et al.*, 1983), the following equation can similarly be derived:

$$\frac{[F]}{[B]} = \frac{1}{n \cdot K_{ass.}[P]} + \frac{[F]}{n \cdot [P]} \quad (8)$$

The Scatchard and the Woolf graphs yielded respectively $K_{ass.} = 236$ and 229 M^{-1} and a number of binding sites n per functional unit with M_r 75000 of 1.08 and 1.09 (correlation coefficients 0.976 and 0.995 for eight experimental points), which verifies the assumption of the binding of one nitrite ion per active site.

The binding curve (Fig. 5), which indicates a true saturation of the binding sites, confirmed the validity of these treatments and yielded a $pK_{ass.}$ value of -2.39 or a $K_{ass.}$ value of 245 M^{-1}. This low binding constant of nitrite to MetHc is also in opposition to the interpretation

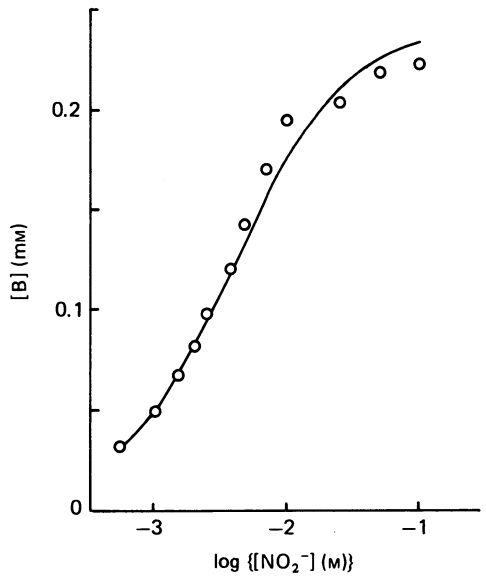

Fig. 5. Plot of the concentration of bound nitrite [B], deduced from e.p.r. signal intensities, measured after 18 h, against the logarithm of free nitrite concentration for *Astacus* MetHc (20.3 mg/ml, 0.48 mM-Cu), prepared with nitrite, in 0.1 M-sodium acetate buffer, pH 5.7, at room temperature

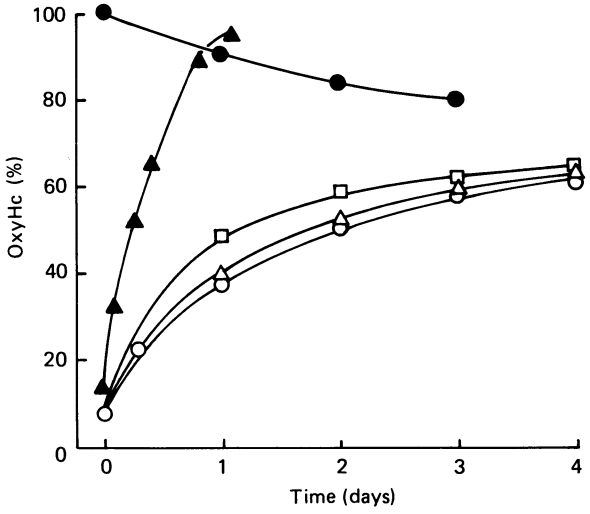

Fig. 6. Regeneration of *Astacus* MetHc, prepared with nitrite, at room temperature as a function of time in 0.1 M-sodium acetate buffer, pH 5.7, at various concentrations of $HONH_2$

○, At 4.7 mM-$HONH_2$ under air; △, at 13.4 mM-$HONH_2$ under air; □, at 23.6 mM-$HONH_2$ under air; ▲, at 13.4 mM-$HONH_2$ under 101 kPa N_2. ●, Decrease of the percentage of OxyHc by the action under air of 13.4 mM-$HONH_2$. Hc concentration was 1.0 mg/ml, 24 μM-Cu.

of NitrosylHc as a nitrite derivative of Semi-MetHc (Hwang & Solomon, 1982), as NitrosylHc is quite stable on dialysis.

Regeneration of MetHc with $HONH_2$

A treatment under air of *Astacus* MetHc, obtained by the action of nitrite, with a freshly prepared solution of $HONH_2$ to a concentration of 4.7 mM in 0.1 M-sodium acetate buffer, pH 5.7, yielded a 60% regeneration of the O_2-binding capacity. The regeneration was not improved at higher $HONH_2$ concentrations (13.4 and 23.6 mM) (Fig. 6). When OxyHc was treated with $HONH_2$ under similar conditions a 20% decrease in the Cu–O_2 band at 338 nm was observed after 72 h (Fig. 6). Under anaerobic conditions the addition of $HONH_2$ did not lower the absorption band at 338 nm, measured on re-oxygenation. An anaerobic treatment with $HONH_2$ of MetHc obtained with nitrite allowed an almost quantitative regeneration after 24 h (Fig. 6). The lower regeneration of MetHc with $HONH_2$ under air may be attributed to traces of H_2O_2, formed in the autoxidation of $HONH_2$ (Kono, 1978), which destroy some active sites.

Astacus MetHc prepared with H_2O_2 (Felsenfeld & Printz, 1959) was regenerated to the extent of about 47% by an anaerobic treatment with $HONH_2$, in contrast with the absence of regeneration under air. It showed a binuclear e.p.r. signal at $g = 2$ in the presence of nitrite in 0.1 M-acetate buffer, pH 5.7, which amounted to 43% of the signal observed with MetHc prepared with nitrite. These data indicate an irreversible damage of the active sites in the one-electron reduction of H_2O_2 by Cu(I). The oxidation of DeoxyHc by nitrite, on the contrary, yields MetHc with undamaged active sites:

$$\mathrm{Cu(I)Cu(I)} + \mathrm{HNO_2} \rightarrow \underset{\mathrm{OH^-}}{\mathrm{Cu(II)}}\mathrm{Cu(I)} + \mathrm{NO^{\bullet}} \qquad (9)$$

$$\underset{\mathrm{OH^-}}{\mathrm{Cu(II)}}\mathrm{Cu(I)} + \mathrm{HNO_2} + 2\mathrm{H^+} \rightarrow \mathrm{Cu(II)}\underset{\mathrm{H\ H}}{\mathrm{Cu(II)}} + \mathrm{NO^{\bullet}} + \mathrm{H_2O} \qquad (10)$$

$$\text{with}\quad \mathrm{Cu(II)}\underset{\mathrm{H\ H}}{\mathrm{Cu(II)}} \overset{pK_a = 6.50}{\rightleftharpoons} \mathrm{Cu(II)}\underset{\mathrm{H^-}}{\mathrm{Cu(II)}} + \mathrm{H^+} \qquad (11)$$

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